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(54) METHODE D'INHIBITION DES AVORTEMENTS D'ORIGINE IMMUNOLOGIQUE
(54) A METHOD OF INHIBITING IMMUNOLOGICALLY MEDIATED ABORTIONS

(57)

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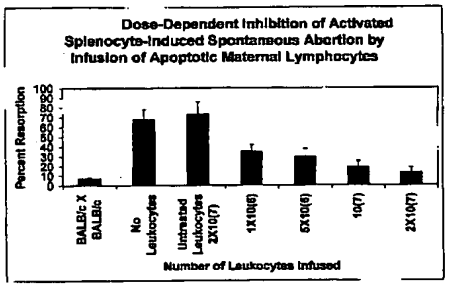

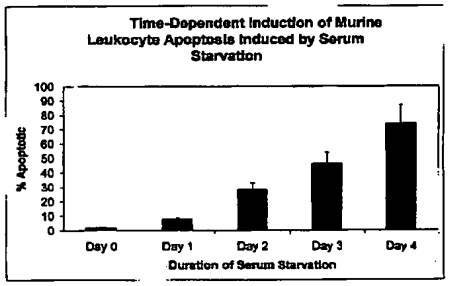

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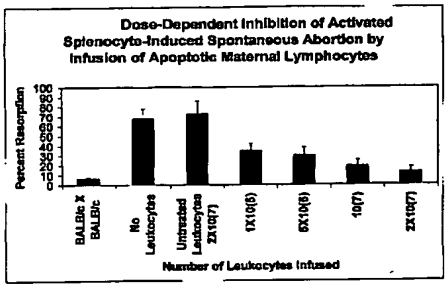

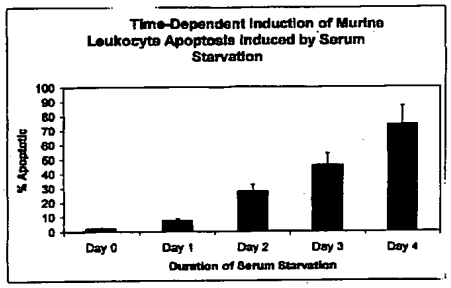

(57) Abrégé/Abstract:
The invention disclosed teaches methods of inhibiting recurrent spontaneous abortion in a mammal through administration of apoptotic leukocytes. Said leukocytes may be derived from maternal or paternal sources. Several methods of apoptotic induction may be used to attain anti-abortogenic erects.

A METHOD OF INHIBITING IMMUNOLOGICALLY MEDIATED ABORTIONS

ABSTRACT

The invention disclosed teaches methods of inhibiting recurrent spontaneous abortion in a mammal through administration of apoptotic leukocytes. Said leukocytes may be derived from maternal or paternal sources. Several methods of apoptotic induction may be used to attain anti-abortogenic effects.

DISCLOSURE

Field of the Invention

The invention teaches methods of modulating the immune system of a mammal in order to prevent spontaneous abortion.

Background

Recurrent Spontaneous Abortion

Recurrent Spontaneous Abortion (RSA) affects 1-2% of women in North America (1). RSA is believed to be caused by abnormal activation of Th1 cells, these cells are generally suppressed in pregnancy. Lin *et al* demonstrated successful murine pregnancy is associated with upregulated production of the Th2 cytokines IL-4, IL-5 and IL-10 in the fetoplacental unit (2). This was postulated to inhibit development of Th1 cells, cells associated with induction of inflammatory responses. The Th2 biasing of immune response by pregnancy can be seen in C57BL/6 mice infected with Leishmania. Normally C57BL/6 mice are resistant to such infection because of a Th1 bias. When these mice are pregnant, due to the Th2 biasing of pregnancy, infection with Leishmania is possible (3). Administration of Th1 cytokines to pregnant mice can increase the rate of abortion (4), whereas administration of Th2 cytokine suppresses it (5). Therapies which prevent RSA, such as paternal lymphocyte infusion are associated with augmentation of Th2 responses (6,7). Therefore it appears that successful pregnancy requires suppression of Th1 and promotion of Th2 immune responses.

Paternal lymphocyte infusion therapy was originally devised by Govallo based on the idea that RSA occurs due to improper recognition of paternal antigens by maternal immune system cells (8). Through immunization with paternal lymphocytes, the mother's immune system will be primed to recognize the

paternal antigens on the placenta, thus immune response will occur. However, Govallo believed that stimulation of antiplacental immune response by injection of paternal lymphocytes would not cause an inflammatory response, but an immune response which would actually help the placenta to grow. A parallel concept was introduced by Prehn in cancer immunology who should that host antitumor immune response in some circumstance can promote tumor growth (9). Although some clinical trials using paternal lymphocyte infusion have reported a decreased number of RSA (10,11), a recent double blind study reported no difference between treated and control groups (12). Nevertheless this practice is still in use today.

Rational exists for the systemic administration of Th2 cytokines in women prone to RSA. Since cytokine like IL-10 can be safely administered, and prevent activation of Th1 immune responses (13). The problem with systemic cytokine therapy is cost associated, as well as the possibility of global immune suppression.

Thus there is a need for treatment of RSA in an economical, safe and effective manner.

Apoptosis

Apoptosis, in contrast to necrosis, is a type of cell death in which membrane integrity of the cell is kept, thus preventing leakage of intracellular organelles to the extracellular mileau (14). The process of apoptosis exists in order to rid the organism of unwanted cells without causing an inflammatory response. In contrast, cells dying by necrosis release various intracellular components causing inflammation and tissue damage. The concept of "danger signals" was originally proposed by Matzinger (15) with the idea that innate signals of danger exist, which instruct the body to launch immune response or to ignore the antigen. Under Matzinger's paradigm, danger is associated with tissue necrosis, whereas lack of danger occurs when cells die by apoptosis. This is exemplified by studies showing that intracellular danger signals are released by cells undergoing necrotic

but not apoptotic cell death. Such danger signals include free-floating heat shock proteins which can activate dendritic cells and macrophages (16, 17). Following with the idea that cells undergoing necrosis cause immune activation through the danger pathway, apoptotic death should inhibit activation of immune response. One method by which apoptotic cells inhibit immune response is through the production of immunosuppressive cytokines such as IL-10. In a study by Gao *et al*, T cells induced to undergo apoptosis secreted IL-10 protein, as well as increased production of IL-10 transcripts. In the same study it was demonstrated that apoptotic T cells can program macrophages to inhibit activation of pro-inflammatory Th1 immune responses (18). In another study, Voll *et al* demonstrated that apoptotic lymphocytes can suppress production of TNF-α and IL-12 from peripheral blood mononuclear cells activated with lipopolysaccharide (LPS) (19).

Conclusion

In light of observations that paternal lymphocytes infusions seem to diminish spontaneous abortion, and that lymphocytes undergoing apoptosis inhibit inflammatory responses, we investigated whether transfer of paternal and maternal origin apoptotic lymphocytes into abortion-prone mice would protect the fetus from immunologically mediated destruction.

Summary of the Invention

The disclosed invention teaches a method of inhibiting abortogenic immune responses through administration of treated blood cells into a pregnant mammal. More specifically, paternal or maternal leukocytes are treated with apoptotic stimuli after which they are introduced into a pregnant female. This process, we postulate, results in the inhibition of inflammatory immune responses, allowing for completion of pregnancy in situations under which the immune response would normally result in fetal destruction.

The invention discloses methods of preparing paternal or maternal leukocytes, in a manner which endows the cells with anti-inflammatory properties. Disclosed is the utility of these cells in inhibiting anti-fetal immune responses. The direct effect of administration of the treated leukocytes on the pregnant female, that is the anti-abortogenic effect, is also disclosed.

Brief Description of the Drawings

Figure I illustrates time-dependent apoptotic effects of serum starvation on murine leukocytes.

Figure II illustrates the dose-dependent antiabortogenic effects of infusing apoptotic paternal cells to a pregnant mother induced to undergo abortion by LPS administration.

Figure III illustrates the dose-dependent antiabortogenic effects of infusing apoptotic maternal cells to a pregnant mother induced to undergo abortion by LPS administration.

Figure IV illustrates the dose-dependent antiabortogenic effects of infusing paternal apoptotic cells into the CBA/J X DBA/2 model of spontaneous abortion.

Figure V illustrates the dose-dependent antiabortogenic effects of infusing maternal apoptotic cells into the CBA/J X DBA/2 model of spontaneous abortion.

Detailed Description of Preferred Embodiments

This invention teaches methods of preventing immunologically-mediated spontaneous abortions through administration of apoptotic lymphocytes. This effect, we believe is mediated through production of soluble and membrane bound components on apoptotic cells which suppress inflammatory immune responses.

It is these inflammatory responses which bring about the demise of the conceptus. In the data displayed apoptosis was achieved through serum starvation of the leukocytes. In other studies we performed similar antiabortigenic effects can be seen by injection of leukocytes in which apoptosis was induced through other methods such as irradiation or treatment with protein synthesis inhibitors. In agreement with previously published studies, the immunomodulatory effects of apoptotic leukocytes are independent of the method by which apoptosis was induced (18, 19).

One embodiment of the invention mends itself applicable to the current practice of immunizing pregnant women at risk of spontaneous abortion with paternal lymphocytes. Paternal leukocytes can be purified from whole blood of the father through the Ficoll™ technique or other methods known to one skilled in the art. Apoptosis can be induced in these cells through serum starvation or by other methods known such as irradiation, treatment with protein synthesis inhibitors, treatment with ozone gas, or treatment with hyperthermia. Quality of the apoptotic cells can be assessed by Annexin-V staining as well as propidium iodide in order to ensure a high percentage of apoptotic but not necrotic cells are administered. Injection of apoptotic cells into the mother can occur via direct intravenous administration using syringe and needle, or continuous drip administration. The same procedure can be carried out using leukocytes derived from the mother.

Detection of cytokines associated with spontaneous abortion such as interferon-γ can serve as an immune marker to guide the number of apoptotic cells injected, as well as the frequency of injection.

Examples

Example I

Induction of Apoptosis by Serum Starvation

BALB/c mice were euthanized by carbon dioxide asphyxiation and spleens were collected under asceptic conditions. Spleens were disassociated with a wire mesh and erythrocytes were lysed with hypotonic buffer. Lysed erythrocytes were removed by centrifugation. Splenocytes were cultured at a concentration of 10^5 cells/well in serum-free RPMI media in 96 well plates in a total volume of 200 µl. At day 0. 1, 2, 3, and 4 apoptosis was assessed using ApoAlert™ Annexin V-FITC apoptosis kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. As illustrated in Figure I a time dependent increase in apoptotic cells was observed.

Example II

Dose-Dependent Inhibition of LPS-Induced Spontaneous Abortion by Infusion of Apoptotic Paternal Leukocytes

Pregnancy was induced in 8 week old BALB/c females by crossing with C57/B6 males. Day zero of pregnancy was established as the day copulatory plug was observed. On day 5 of pregnancy a single injection of 4 µg lipopolysaccharide from *Escherichia coli* (LPS: Sigma Chemicals, St. Louis, MO), was administered in 100 µl of saline through the intraperitoneal route. On day 5 an injection of apoptotic paternal (C57/B6) leukocytes was administered intravenously through the tail vein. Apoptotic leukocytes were induced by serum starvation for 4 days as described in Example I. Mice were sacrificed on day 12 of pregnancy. As shown in Figure II administration of fresh splenocytes (Untreated leukocytes) did not inhibit LPS-induced abortions whereas injection of apoptotic leukocytes induced a dose-dependent inhibition of abortions. Abortions were quantified as

percent resorption identified by morphological appearance. Each bar represents the average (±Standard Deviation) of 7 mice.

Example III

Dose-Dependent Inhibition of LPS-Induced Spontaneous Abortion by Infusion of Apoptotic Maternal Leukocytes

Pregnancy was induced in 8 week old BALB/c females by crossing with C57/B6 males. Day zero of pregnancy was established as the day copulatory plug was observed. On day 5 of pregnancy a single injection of 4 μg lipopolysaccharide from *Escherichia coli* (LPS: Sigma Chemicals, St. Louis, MO), was administered in 100 μl of saline through the intraperitoneal route. On day 5 an injection of apoptotic maternal (BALB/c) leukocytes was administered intravenously through the tail vein. Apoptotic leukocytes were induced by serum starvation for 4 days as described in Example I. Mice were sacrificed on day 12 of pregnancy. As shown in Figure III administration of fresh splenocytes (Untreated leukocytes) did not inhibit LPS-induced abortions whereas injection of apoptotic leukocytes induced a dose-dependent inhibition of abortions. Abortions were quantified as percent resorption identified by morphological appearance. Each bar represents the average (±Standard Deviation) of 7 mice.

Example IV

Dose-Dependent Inhibition Spontaneous Abortion in The CBA/J X DBA/2 Model by Infusion of Apoptotic Paternal Lymphocytes.

CBA/J female mice were bred with DBA/2. Day zero of pregnancy was established as the day copulatory plug was observed. On day 5 an injection of apoptotic paternal (DBA/2) leukocytes was administered intravenously through the tail vein. Apoptotic leukocytes were induced by serum starvation for 4 days

as described in Example I. Mice were sacrificed on day 12 of pregnancy. As shown in Figure IV administration of fresh splenocytes (Untreated leukocytes) did not inhibit LPS-induced abortions whereas injection of apoptotic leukocytes induced a dose-dependent inhibition of abortions. Abortions were quantified as percent resorption identified by morphological appearance. Each bar represents the average (±Standard Deviation) of 7 mice.

Example V

Dose-Dependent Inhibition Spontaneous Abortion in The CBA/J X DBA/2 Model by Infusion of Apoptotic Maternal Lymphocytes.

CBA/J female mice were bred with DBA/2. Day zero of pregnancy was established as the day copulatory plug was observed. On day 5 an injection of apoptotic maternal (CBA/J) leukocytes was administered intravenously through the tail vein. Apoptotic leukocytes were induced by serum starvation for 4 days as described in Example I. Mice were sacrificed on day 12 of pregnancy. As shown in Figure V administration of fresh splenocytes (Untreated leukocytes) did not inhibit LPS-induced abortions whereas injection of apoptotic leukocytes induced a dose-dependent inhibition of abortions. Abortions were quantified as percent resorption identified by morphological appearance. Each bar represents the average (±Standard Deviation) of 7 mice.

The invention discloses a method of inhibiting immunologically mediated abortions in a mammal. It is to be understood that the above examples are presented only for clarity and that the invention may take other embodiments as practiced by one skilled in the art.

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CLAIMS

1. A method of preventing recurrent spontaneous abortion in a mammal through

(a) Withdrawing leukocytes from a donor
(b) Inducing apoptosis of the donor leukocytes
(c) Administration of the donor leukocytes to a female susceptible to RSA

2. The method of claim I in which leukocyte defines a purified population of neutrophils, monocytes, dendritic cells, T cells or B cells.

3. The method of claim I in which leukocyte defines a combination of neutrophils, monocytes, dendritic cells, T cells and B cells.

4. The method of claim I where apoptosis is induced through ultraviolet irradiation, X-irradiation, γ-irradiation, serum starvation or crosslinking of Fas.

5. The method of claim I where the apoptotic cells are used to prevent recurrent spontaneous abortion in a mammal.

6. The method of claim I where the donor can be maternal or paternal in reference to the pregnancy.

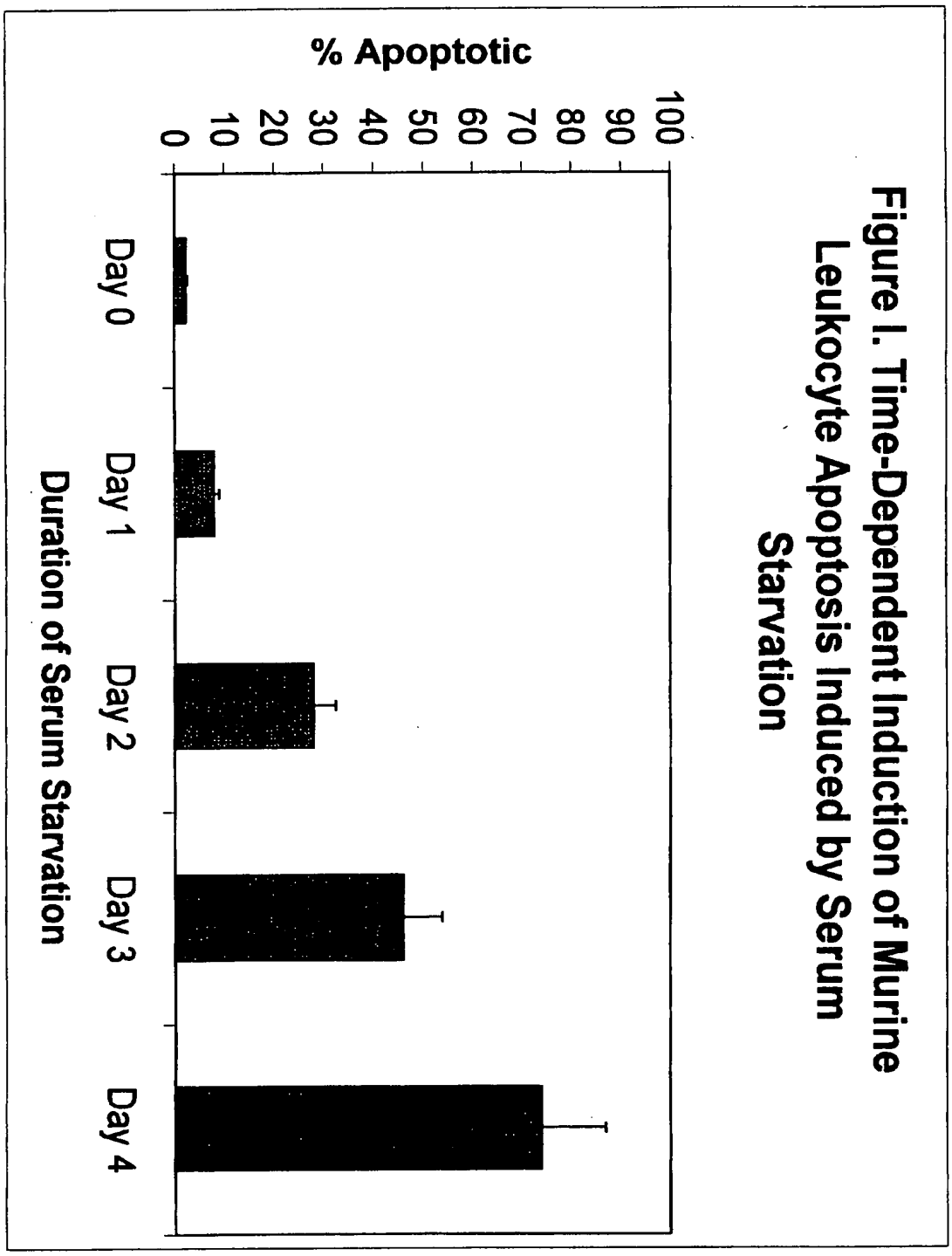

Figure I. Time-Dependent Induction of Murine Leukocyte Apoptosis Induced by Serum Starvation

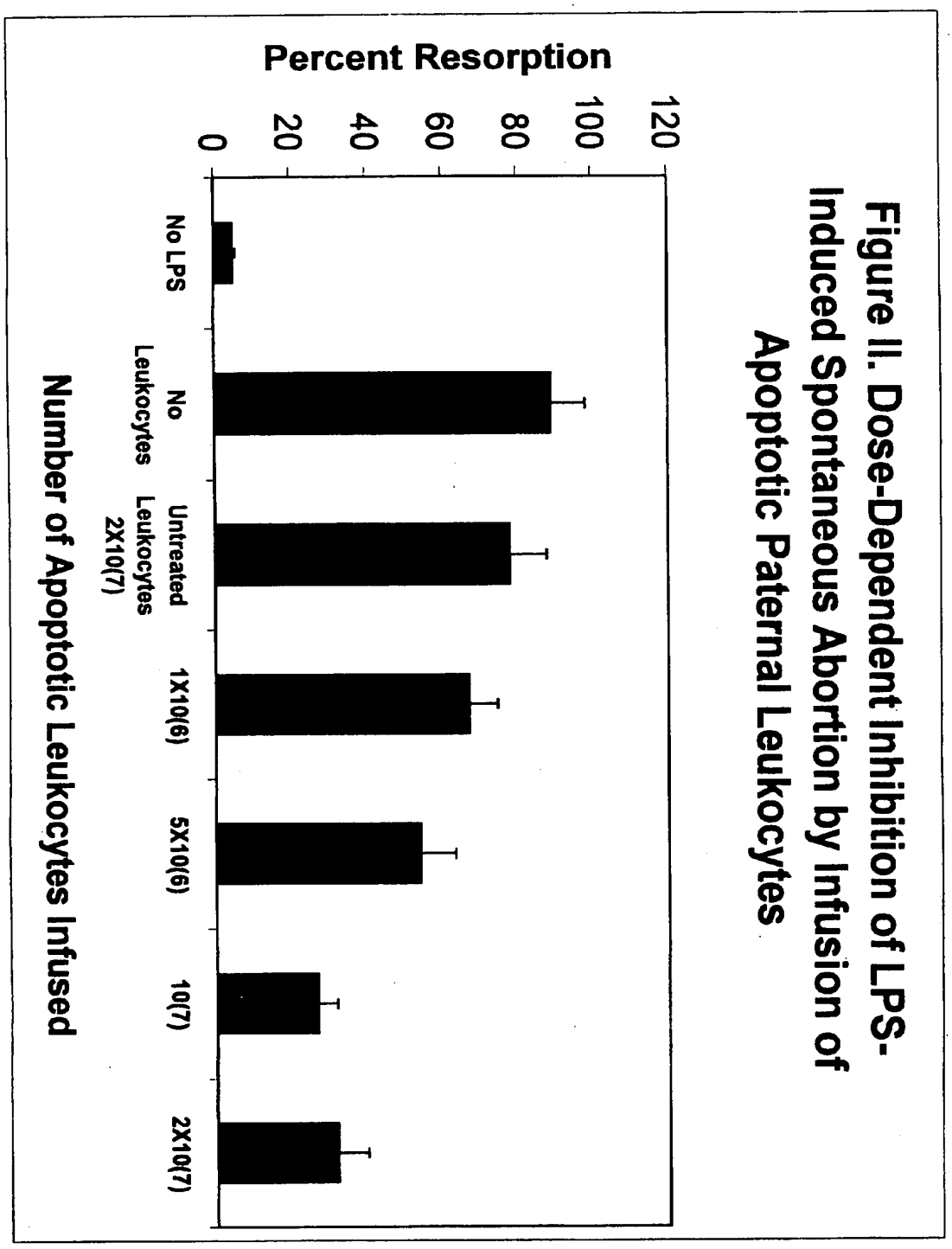

Figure II. Dose-Dependent Inhibition of LPS-Induced Spontaneous Abortion by Infusion of Apoptotic Paternal Leukocytes

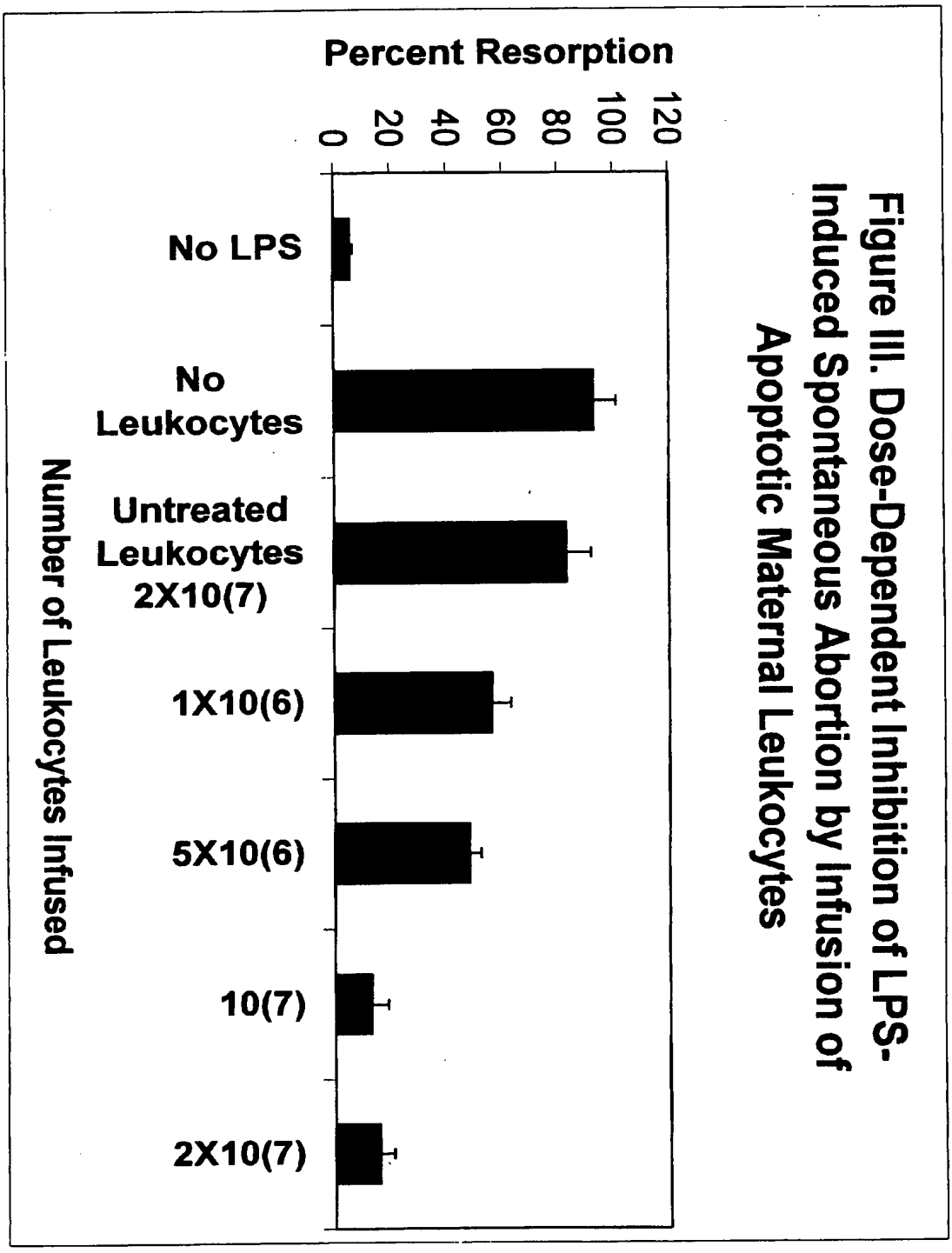

Figure III. Dose-Dependent Inhibition of LPS-Induced Spontaneous Abortion by Infusion of Apoptotic Maternal Leukocytes

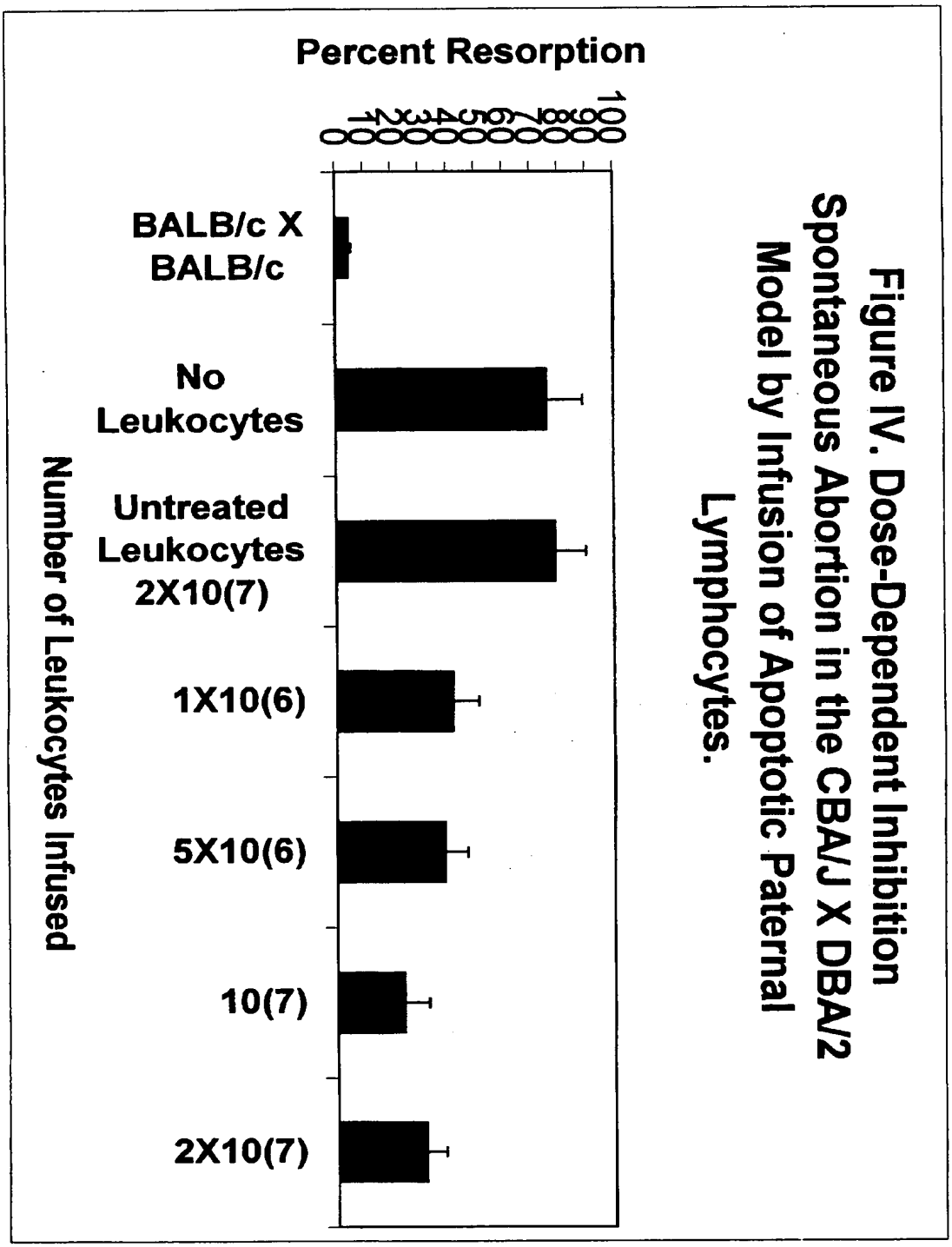

Figure IV. Dose-Dependent Inhibition Spontaneous Abortion in the CBA/J X DBA/2 Model by Infusion of Apoptotic Paternal Lymphocytes.

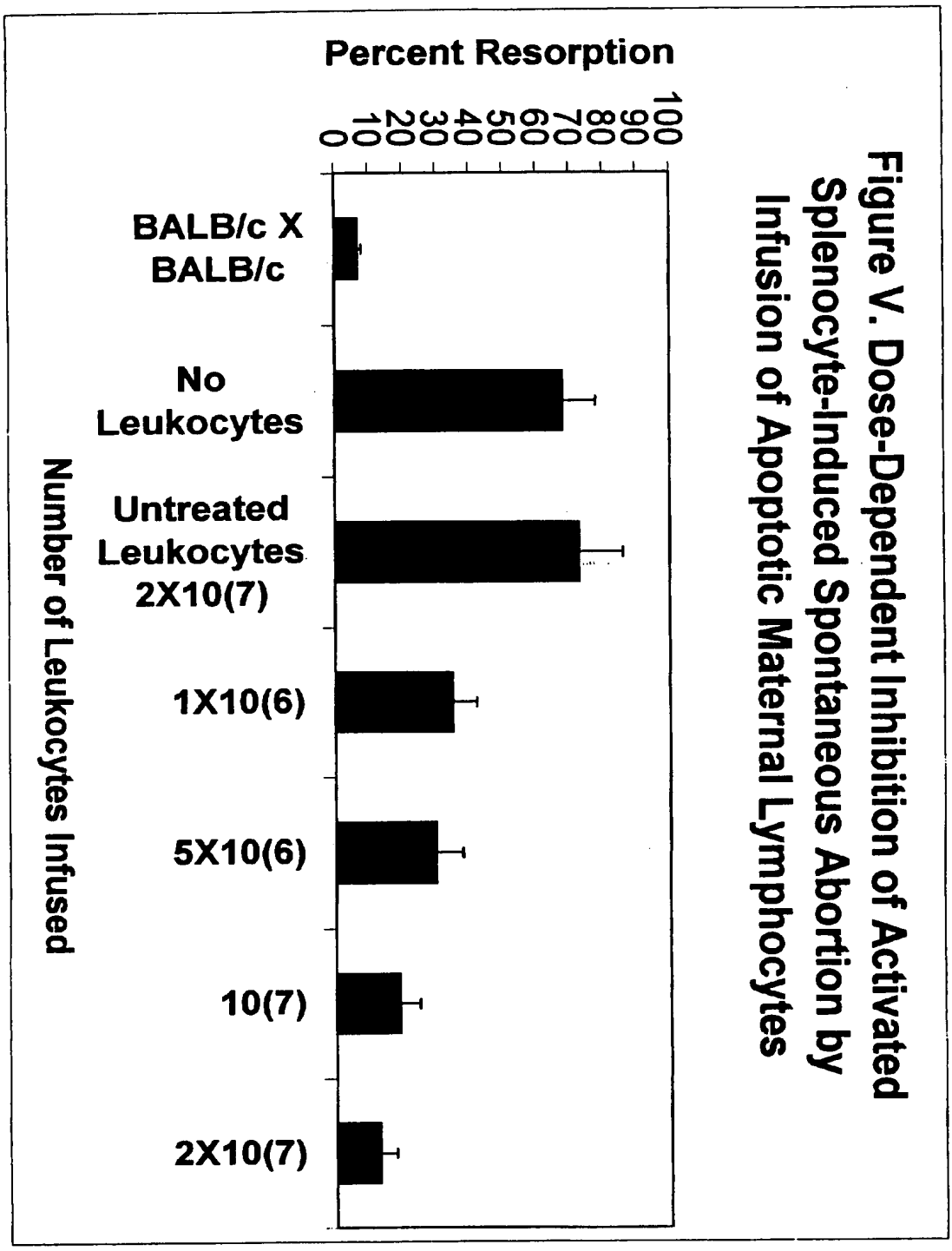

Figure V. Dose-Dependent Inhibition of Activated Splenocyte-Induced Spontaneous Abortion by Infusion of Apoptotic Maternal Lymphocytes